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(54) Title: A SPRAY–DRIED BACTERIOCIN POWDER WITH ANTI–MICROBIAL ACTIVITY

(57) Abstract

The production of a spray–dried bacteriocin lacticin 3147 powder is described. The powder is shown to have effective anti–microbial activity in a range of foodstuffs, namely infant milk formulations, powdered soup, yoghurt and cottage cheese. Increased anti–microbial activity was demonstrated when the lacticin 3147 powder was used in conjunction with increased hydrostatic pressure. The process comprises: inoculating a medium with a lacticin 3147–producing strain of bacteria, fermenting the inoculated medium, adjusting the pH of the fermentation to 6.3–6.7, inactivating the bacterial fermentate and evaporating the fermentate.

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A spray-dried bacteriocin powder with anti-microbial activity

The present invention relates to a spray-dried bacteriocin powder with anti-microbial activity, and to a method of producing the powder. In particular, the invention relates to a lacticin 3147 spray-dried powder.

Prior Art

The elimination of food spoilage and pathogenic organisms has become the focus of much research since, in terms of individuals affected and the cost of treatment, food-borne illnesses have an enormous impact. It has been estimated that microbial pathogens in food cause 6.5 - 33 million cases of human illness annually in the U.S., at a cost of between $2.9 - $6.7 billion dollars (2), with Gram-positive food-borne pathogens accounting for between 25 - 55% of the costs. In recent years, consumer demand for fresh minimally processed safe food, in addition to concern over the use of chemical preservatives in foods, has prompted substantial interest in the application of biopreservatives. Bacteriocins produced by lactic acid bacteria are seen as alternatives to traditional preservatives for ensuring food safety and potential applications in foods have been readily identified (21).

Nisin, a bacteriocin produced by certain strains of Lactococcus lactis, has been used successfully to control food spoilage, in a number of different foods, including cheeses, canned goods and dairy desserts (10). However, its use is subject to certain restrictions. It is most effective in foods with acidic pH (below pH 6.0) and low protein and fat content. It is poorly soluble above pH 6.0 and as such has limited effectiveness in many foods. A powdered form of nisin, Nisaplin (Aplin and Barrett, Towbridge, Wiltshire, U. K.) has been developed and is used for the preservation of foods.

In addition to the development of Nisaplin, other powdered bacteriocin-containing agents have been developed for the preservation of foods. *Propionibacterium freundenreichii* subsp. *shermanni* is used to produce Microgard (Wesman Foods, Inc., Beaverton, OR.) by pasteurisation and drying of propionibacteria-fermented skim milk. It is estimated to be used in approximately one third of all cottage cheese made in the US and is said to be inhibitory to most Gram-negative bacteria and some fungi (4). The active agents in Microgard include propionic acid, acetic acid, diacetyl, lactic acid and a heat-stable peptide of approximately 700 daltons which is considered to be the most active component.

Lacticin 3147 is a bacteriocin produced by *L. lactis* DPC3147 which has a similar host range to that of nisin, in that it is inhibitory to a wide range of Gram-positive organisms, including *Listeria*, *Clostridium* spp., *Enterococcus*, *Staphylococcus* and *Streptococcus* (17). Given that many of these organisms have been identified as agents of food spoilage and pathogenesis, the development of a lacticin 3147-based system for control of these organisms has obvious attractions. This may be achieved in two ways. The first involves the use of starter cultures (including transconjugants) which produce lacticin 3147, and can be used in food fermentations where these strains can be substituted for the original starter cultures. The genetic determinants for lacticin 3147 are encoded on a 60.2 kb plasmid, pMRC01 which has been fully sequenced (6) and which has been mobilised to a number of cheese starter cultures (3). Lacticin 3147 is the subject of PCT Application No. PCT/IE96/00022, published as WO 96/32482.

Recently, it has been shown that a lacticin 3147 producing transconjugant can inhibit *Listeria monocytogenes* in Cottage cheese (13). This starter has also been used to control the proliferation of non-starter lactic acid bacteria in Cheddar cheese. The second approach to improving food safety through the use of lacticin 3147 involves the development of a spray-dried form of the bacteriocin. The advantage of such a bio-active powder is that it could be applied as a food ingredient in a variety of foods. However, it is not at all apparent that the bacteriocin is robust enough to withstand spray-drying and there was the possibility that spray-drying would result in a significant loss in bacteriocin activity.

Object of the invention

The object of the invention is to provide a lacticin 3147-enriched food ingredient for incorporation into foodstuffs. In particular, it is an object to provide a spray-dried lacticin 3147 powder. It could not be predicted that such a spray-dried powder could be produced since spray-drying could have caused heat denaturation of the bacteriocin, bearing in mind that lacticin 3147 is composed of two peptides, both of which are required for activity. Furthermore, dehydration could irreversibly inactivate the bacteriocin.

Described herein is a whey based bio-active powder, with effectiveness in controlling two representative pathogens, *L. monocytogenes* and *Staphylococcus aureus*, in buffer at both neutral and acidic pH. Also described is its effectiveness in controlling *L. monocytogenes* in an infant milk formulation and other foodstuffs. However, it will be apparent to those skilled in the art that the bacteriocin-powder of the invention need

not be dairy based and that it would also be possible to produce a spray-dried bacteriocin based, for example, on other powders, synthetic materials or the like.

Summary of the Invention

According to the present invention there is provided a process for the production of spray-dried lacticin 3147 powder comprising:

(a) inoculating a medium with a lacticin 3147-producing strain of bacteria;
(b) fermenting the inoculated medium;
(c) adjusting the pH of the fermentation to pH 6.3 to 6.7;
(d) inactivating the bacterial fermentate;
(e) evaporating the fermentate of step (d).

The medium which may be inoculated with the bacteria can be selected from milk or dairy-based powders including demineralised whey powder, reconstituted skimmed milk powder, whey protein concentrate powder, pasteurised milk, Cheddar cheese whey, or synthetic laboratory media such as LM17 or TY broth or the like.

Preferably the inoculated medium is fermented at about 30°C for about 6 to 24 hours.

Preferably the pH of the fermentation is adjusted to about 6.5.

Suitably, the bacterial fermentate is inactivated by pasteurisation or treating at ultra-high temperature.

Suitably, if the fermentate is pasteurised, it is pasteurised at about 72°C for about 15 seconds.

Preferably the inactivated fermentate is evaporated at about 6°C to about 40% total solids.

The concentrate of step (e) may then be cooled to about 32°C, seeded with lactose at about 0.1% w/w and allowed to crystallise at a cooling rate of about 1°C per hour.

The crystallised concentrate is then spray-dried by methods known in the art.

The invention also provides a spray-dried lacticin 3147 powder which has the ability to inhibit organisms which are not resistant to lacticin 3147 and which may suitably have an activity of about 40,240 au (arbitary units)/per ml.

The invention also provides a food product comprising a spray-dried lacticin 3147 powder as defined above. The food product may be an infant milk formulation, a sauce, mayonnaise, a dessert, a yoghurt, a custard, a tinned food product such as a tinned vegetable or tinned meat product, a soup, a bakery product or similar products.

The food product may further have been subjected to increased hydrostatic pressure during processing, suitably at a pressure of about 150 to 800 MPa.

FIGURE LEGENDS

The present invention will now be described in greater detail with reference to the accompanying drawings in which:

Figure 1. (A) Growth of *L. lactis* DPC3147 and lacticin 3147 production in 10% reconstituted demineralized whey powder at 30°C, in pH controlled and uncontrolled conditions. (◇) cfu/ml with no pH control imposed, (□) cfu/ml at constant pH of 6.0, (○) cfu/ml at constant pH of 6.5 and (○) cfu/ml at constant pH of 7.0. (▨) AU/ml with no pH control, (▨) AU/ml at constant pH of 6.0, (▨) AU/ml at constant pH of 6.5 and (▨) AU/ml at constant pH of 7.0.
(B) Inhibitory activity of lacticin 3147 against L. lactis HP when (a) grown with no pH control and when (b) grown at a constant pH of 6.5.

Figure 2. Schematic diagram of temperature profile and lacticin 3147 activity during the manufacturing of lacticin 3147 powder.

Figure 3. Effect of lacticin 3147 powder on the viability of Listeria monocytogenes Scott A in buffer at 30°C (A) at pH 5 and (B) at pH 7. (◇) no addition, (□) addition of 10% lacticin 3147 powder.

Figure 4. Effect of lacticin 3147 powder on the viability of Staphylococcus aureus 10 in buffer at 30°C (A) at pH 5 and (B) at pH 7. (◇) no addition, (□) addition of 15% lacticin 3147 powder.

Figure 5. Effect of lacticin 3147 powder on the viability of *L. monocytogenes* Scott A when used as a component of infant milk formula. (□) 15% lacticin powder, (△) 10% lacticin powder, 5% infant milk powder, (○) 5% lacticin powder, 10% infant milk powder, (◇) 15% infant milk powder.

Figure 6. Effect of lacticin 3147 powder (10%) on the viability of *Listeria monocytogenes* Scott A in yoghurt. (◇) no lacticin 3147 added, (□) 10% lacticin 3147 added. The 10% here refers to 10g lacticin 3147 powder added to 90g yoghurt.

Figure 7. Effect of lacticin 3147 powder (10%) on the viability of *Listeria monocytogenes* Scott A in cottage cheese. (◇) no lacticin 3147 added, (□) 10% lacticin 3147 added. The 10% here refers to 10g lacticin 3147 powder added to 90g cottage cheese.

Figure 8. Effect of lacticin 3147 powder (10%) on the viability of *Bacillus cereus* in (packet) soup.
(◇) no lacticin 3147 added,
(□) 1% lacticin 3147 added,
(△) 5% lacticin 3147 added,
(○) 10% lacticin 3147 added.
The 1, 5, 10% here refers to 1, 5 or 10g lacticin 3147 powder added to 99, 95 or 90g packet soup powder, then reconstituted to the manufacturers instructions.

Figure 9. Effect of lacticin 3147 powder (10%) on the viability of *Listeria monocytogenes* Scott A in (packet) soup.
(◇) no lacticin 3147 added,
(□) 1% lacticin 3147 added,
(△) 5% lacticin 3147 added,
(○) 10% lacticin 3147 added.
The 1, 5, 10% here refers to 1, 5 or 10g lacticin 3147 powder added to 99, 95 or 90g packet soup powder, then reconstituted to the manufacturers instructions.

Figure 10. The effect of increasing pressures on the activity of lacticin 3147, (a) atmospheric pressure, (b) 200 MPa, (c) 400 MPa, (d) 600 MPa and (e) 800 MPa.

Figure 11. The effect of high pressure and lacticin 3147 on *L. innocua* DPC1770 viability.

Example

MATERIAL AND METHODS

Bacterial strains and culture conditions

The bacteriocin producer *L. lactis* subsp lactis DPC3147 and the sensitive indicator strain *L. lactis* subsp lactis HP were routinely grown at 30°C in M17 (20; Oxoid Ltd., Basingstoke, Hampshire, England) supplemented with 0.5% (w/v) lactose. Other indicator strains used included *L. monocytogenes* Scott A grown in Trypicase Soy Broth (TSB, Becton Dickinson and Co., Cockeysville, MD21030, USA) supplemented with 0.6% (w/v) yeast extract (Oxoid), and *Staphylococcus aureus* 10 (DPC culture collection, Moorepark, Fermoy, Co. Cork, Ireland) grown in Brain Heart Infusion broth (BHI, Oxoid), both at 37°C. Solid media was prepared by the addition of 1% bacteriological agar (Oxoid).

A number of different media were investigated for the production of lacticin 3147. These were made as 10% (w/v) solutions, apart from pasteurised whole milk and Cheddar cheese whey. The 10% solutions were prepared from demineralized whey powder (95% demineralized), reconstituted skimmed milk powder (Dairygold, Mitchelstown, Co. Cork, Ireland) and whey protein concentrate powder (WPC35, 35% protein in dry matter, Moorepark Technology Ltd., Moorepark, Fermoy, Co. Cork, Ireland). The whey based solutions were sterilised by heating to 95°C for 30 minutes. The skimmed milk powder solution was sterilised by autoclaving for 5 min at 121°C.

Bacteriocin assay and activity determination

Bacteriocin activity was determined by the agar well diffusion assay as described by Parente and Hill (15). Molten agar was seeded with an indicator strain and dispensed into petri dishes. Wells of approximately 4.6mm in diameter were bored in the agar and a 50µl volume of a two fold serial dilution of a bacteriocin preparation was dispensed into each well. Bacteriocin solution was prepared by centrifuging the culture

and heat treating the supernatant at 70°C for 10 minutes prior to carrying out the dilution series. The plates were then incubated at either 30°C or 37°C, depending on the indicator strain used. Bacteriocin activity was calculated as the inverse of the last dilution that gave a definite zone of clearance after overnight incubation. Activity units (AU) were expressed per milliliter (1/dilution, x 20).

Controlled pH fermentations.

Controlled pH fermentations were carried out over a 24 hour period, with slow agitation (approximately 20 rpm) at 30°C. A 1% inoculum of DPC3147 was used to inoculate 100ml of growth media. The pH of the growth media was kept at a constant value by the addition of 1.0 M NaOH on demand via a 718 STAT Titrino (Metrohm, Ireland). Cell counts and bacteriocin activity determinations were carried out at hourly intervals for the first 10 hours, and a final sample was taken after 24 hours.

Production of a spray dried lacticin 3147 powder.

A 170 L volume of demineralized whey powder (10% total solids) was inoculated with 1% DPC3147 and the pH of the 24 hour fermentation was controlled by the addition of 2.5M NaOH on demand (pH 6.5). The fermentate was then pasteurised at 72°C for 15 sec using an APV SSP pasteurizer (APV, Silkborg, Denmark). The pasteurised fermentate was then evaporated at 60°C to 40% total solids using a single effect falling film evaporator (Anhydro model F1 Lab). The resulting concentrate was cooled to 32°C, seeded with lactose (0.1% w/w) and allowed to pre-crystallize overnight at a cooling rate of 1°C per hour. The pre-crystallized concentrate was then spray-dried using nozzle atomization in an Anhydro spray drier (Anhydro model Lab 3) at an air inlet temperature of 190°C and a 90°C outlet temperature. The powder was aliquoted, sachet packed in foil-lined sample bags and stored at 4°C. Bacteriocin activity was assessed at each step during the process.

Effect of lacticin 3147 powder against pathogens in buffer

Sensitive cells were grown to mid-exponential phase, washed and resuspended at approximately 10^7 - 10^8 cfu/ml in 2.5 mM sodium phosphate buffer, pH 7.0 or pH 5.0, and 2.5 mM sodium phosphate buffer, pH 7.0 or pH 5.0 supplemented with 10mM glucose. Lacticin 3147 powder was added (at different concentrations depending on the sensitive strain under investigation) and samples were taken at appropriate time intervals over a 3 hour period to determine the viable cell count.

Effect of lacticin 3147 powder against *L. monocytogenes* in an infant milk formulation

Lacticin 3147 powder was added to a commercially available infant milk formula, [ingredients listed as follows: demineralized whey powder, vegetable oils, lactose, skimmed milk, calcium carbonate, potassium citrate, calcium chloride, sodium citrate, magnesium chloride, vitamin C, emulsifier (soya lecithin), taurine, potassium hydroxide, iron sulphate, zinc sulphate, vitamin E, nicotinamide, pantothenic acid, vitamin A, copper sulphate, citric acid, thiamin, vitamin B_6, (carotene, manganese sulphate, potassium iodide, folic acid, vitamin K, sodium selenite, vitamin D, biotin). Manufacturers instructions indicate that the final liquid for infant consumption is a 15% solution (w/v). In experiments the 15% (w/v) infant milk powder was replaced with either 5% (w/v) lacticin powder and 10% (w/v) infant milk powder, or with 10% (w/v) lacticin powder and 5% (w/v) infant milk powder. *L. monocytogenes* cells were grown to mid-exponential phase, washed and resuspended at approximately 10^4 cfu/ml in the various infant milk formulations at 30°C and samples were taken at appropriate time intervals over a 3 hour period to determine the viable cell count.

Preparation of lacticin 3147 for use in high pressure inactivation studies

For the inactivation of *Staph. aureus* ATCC6538 a liquid preparation of lacticin 3147 was prepared using hydrophobic adsorption chromatography. For studies on inactivation of *L. innocua* DPC1770 a food grade powdered preparation of lacticin 3147 was manufactured as described above with the following modification; a 1% demineralised whey powder solution was fermented with *L. lactis* subsp. *lactis* DPC3147 under pH controlled conditions of pH 6.0 for 18 hours.

Activity of both lacticin 3147 preparations was determined by the agar well diffusion assay as described by Parente and Hill (15). Molten agar was seeded with the indicator strain *L. lactis* subsp. *lactis* HP and dispensed into petri dishes. Wells of approximately 6.0 mm in diameter were bored in the agar and a 50µl volume of a two fold serial dilution of a bacteriocin preparation was dispensed into each well. The plates were then incubated at 30°C. Bacteriocin activity was calculated as the inverse of the last dilution that gave a definite zone of clearance after overnight incubation. Activity units (AU) were expressed per milliliter (1/dilution, x 20). Activity may also be expressed as zone diameter (mm), where the diameter of the first zone (neat, undiluted sample) of the dilution series is recorded.

Effect of high pressure on *Staph. aureus* ATCC6538 and *L. innocua* DPC1770 viability

Staph. aureus ATCC6538 cells were resuspended in 10% RSM and aliquoted into sterile 700µl PCR eppendorfs prior to placing in sterile stomacher bags (Seward Ltd., London, UK). Ten millilitre volumes of *L. innocua* DPC1770 cells were resuspended in 20% reconstituted demineralised whey powder aliquoted into sterile stomacher bags. Samples were individually vacuum sealed prior to placing in the pressure vessel (Stansted Fluid Power Ltd., Stansted, England). The vessel consisted of a stainless-steel cylinder (37mm diameter x 300mm height) filled with a 15% (v/v) caster oil in ethanol solution which acts as the hydrostatic pressurisation medium. Samples were treated for 30 min at 25°C in the pressure range 150 to 600 MPa, in addition to a control sample being held at atmospheric pressure (0.1 MPa). All experiments were carried out in duplicate. The chamber temperature was determined by means of a thermoregulating system which circulated to maintain the chamber temperature.

Effect of high pressure on lacticin 3147 activity

To determine the effect of high pressure on lacticin 3147 activity, reconstituted lacticin 3147 powder and aliquots of liquid lacticin 3147 were vacuum sealed and exposed to pressures ranging from 100 to 800 Mpa as described above. Pressurised and non-pressurised solutions of lacticin 3147 were heat treated at 80°C for 10 minutes prior to carrying out activity determination by the well diffusion assay using *L. lactis* HP as an indicator strain.

RESULTS

The objective of this research was to develop a powdered form of lacticin 3147 suitable for use as an ingredient which could help in the control of undesirable micro-organisms in foods. Following the optimization of lacticin 3147 production a scale-up fermentation was carried out and the fermentate was spray-dried to form a bacteriocin-rich powder. This powder was assessed in both a buffer and an infant milk food system for its ability to inhibit pathogens.

Lacticin 3147 production in various media

Following inoculation of DPC3147 (1%) and overnight incubation at 30°C, lacticin 3147 activity was assessed in a number of different growth media. Most of the media were dairy based, but two synthetic media were also included (LM17 and TY). Results of production of lacticin 3147 (see Table 1) demonstrated that activity was high in almost all the dairy based media (1,280 to 2,560 AU/ml) apart from WPC35 (320 AU/ml). Highest levels of lacticin 3147 activity were found in Cheddar cheese whey, whole milk and LM17 (2,560 AU/ml). Both 10% reconstituted demineralized whey powder and 10% reconstituted skimmed milk powder gave activity of 1,280 AU/ml. Lower levels of lacticin 3147 activity were observed in TY broth (640 AU/ml).

Since demineralized whey powder is a commercially and readily available, and good lacticin 3147 activity was observed in this media, further investigations into the optimization of lacticin 3147 production in demineralized whey powder was carried out.

Optimization of lacticin 3147 production in 10% reconstituted demineralized whey powder

Bacteriocin production and viable cell counts in pH-controlled and pH-uncontrolled fermentations revealed that increased levels of lacticin 3147 could be produced by maintaining the pH of the growth media constant, at pH 6.5 (Figure 1). Levels of bacteriocin activity reached 10,240 AU/ml in 10% reconstituted demineralized whey powder when the pH of the growth media was held constant at pH 6.5 (Figure 1B (a)) compared to 640 AU/ml when no pH control was imposed (Figure 1B (b)). At both pH 6.0 and pH 7.0 lacticin activity reached 5120 AU/ml. Results of viable cell counts over a 24 hour period indicated that increased bacteriocin activity corresponded to higher cell densities. Without pH control viable cell counts reached 1 x 10^9 cfu/ml, whereas when the pH of the growth media was maintained at a constant pH of 6.5 viable cell counts reached 3.8 x 10^9 cfu/ml (Figure 1A). With pH control at 6.0 and 7.0 viable cell counts reached 2.5 x 10^9 cfu/ml.

Production of lacticin 3147 powder

A spray-dried lacticin 3147 preparation was manufactured as described in materials and methods. During the manufacturing process bacteriocin activity was assessed at each step, using *L. lactis* HP as the indicator strain (Figure 2). Following the pH controlled fermentation (in 10% reconstituted demineralized whey powder) bacteriocin activity was 10,240 AU/ml. The fermentate was subjected to pasteurisation to inactivate the bacteriocin producing culture DPC3147. Pasteurisation had no effect

on bacteriocin activity (Figure 2). Evaporation (from 10% total solids to 40% total solids) led to a concentration of the fermentate and resulted in an increase in bacteriocin activity to 40,960 AU/ml. Following overnight crystallisation, the activity of the concentrate remained stable. Spray drying of the concentrate resulted in the production of an active powder. When resuspended at a concentration of 50mg/ml (5% solids) the spray dried powder contained 5,120 AU indicating that the activity of the lacticin powder was 102,400 AU/g (100% solids). Lacticin 3147 activity expressed as AU/g of dry matter remained constant throughout manufacture at 102,400 AU/g, indicating that no loss in bacteriocin activity occurred during processing.

The inhibitory activity of the bacteriocin-enriched powder was attributed to the action of lacticin 3147 rather than other fermentation metabolites such as lactic acid, since it inhibited a sensitive *L. lactis* MG1614, but did not show any inhibitory effect against a transconjugant containing the pMRC01 plasmid.

Effect of lacticin 3147 powder on pathogens

The lacticin 3147 enriched demineralized whey powder (lacticin 3147 powder) was investigated for its ability to inhibit two food-borne pathogens. The inhibitory effect of the powder was investigated at pH 5 and at pH 7, in the presence and absence of 10mM glucose. The effectiveness of a 10% (w/v) solution of lacticin 3147 powder against mid-exponential growth phase cells of *L. monocytogenes* Scott A demonstrated that approximately a 3.3 log kill (99.95% kill) could be achieved at pH 5 within 3 hours at 30°C (Figure 3A). Killing of *L. monocytogenes* Scott A with a 10% (w/v) solution of lacticin powder was slightly more effective at pH 7 (Figure 3B). A 3.8 log kill (99.98% kill) was observed within 3 hours at 30°C.

S. aureus 10 was found to be more resistant than *L. monocytogenes* Scott A to the action of the lacticin enriched powder, for this reason a 15% solution of the powder was used. The effectiveness of a 15% (w/v) solution of lacticin 3147 powder against mid-exponential phase cells of *S. aureus* 10 resulted in approximately a 1.1 log kill (90.4% kill) at pH 5 within 3 hours at 30°C (Figure 4A). The killing effect of a 15% solution (w/v) of lacticin powder increased dramatically at pH 7, where almost a 4 log kill (99.98% kill) of *S. aureus* 10 was observed within 3 hours at 30°C (Figure 4B). The inclusion of 10mM glucose resulted only slight increases in the level of cell deaths for either *L. monocytogenes* Scott A or *S. aureus* 10 (results not shown).

Effect of lacticin 3147 powder against *L. monocytogenes* Scott A in an infant milk formulation

To evaluate the effectiveness of the lacticin 3147 powder in a food system experiments were carried out in an infant milk formula, since this is an example of a food destined for a high-risk consumer which contains demineralized whey powder as a major constituent. Results indicated greater that a 99% kill of *L. monocytogenes* Scott A resulted when part of the infant milk formulation was substituted with either two thirds (10% lacticin powder and 5% infant milk powder) or one third lacticin 3147 powder (5% lacticin powder and 10% infant milk powder) (Figure 5). Counts here were reduced from approximately 7×10^4 cfu/ml to 3×10^1 cfu/ml within 3 hours at 30°C. In the control culture with no lacticin 3147 powder present counts increased from approximately 10^4 cfu/ml to approximately 10^5 cfu/ml within the same time period.

Application of lacticin 3147 powder in a range of foods

Powdered lacticin 3147 has been assessed for the inhibition of food spoilage and pathogenic micro-organisms in a number of food systems including infant food formula, powdered soup, cottage cheese and natural yoghurt. The following are specific examples of the use of lacticin 3147 to inhibit pathogens in food systems.

The ability of the lacticin 3147 powder to inhibit *Listeria monocytogenes* Scott A was initially investigated in an infant milk formulation as described above. To further investigate the inhibitory effect of the lacticin 3147 powder, inactivation trials were carried out against a number of different micro-organisms in natural yoghurt, cottage cheese and reconstituted powdered soup, with pHs of 4.5, 4.4 and 6.6 respectively.

The effect of 10% lacticin 3147 powder on the inhibition of *Listeria monocytogenes* Scott A (10^4 cfu/ml) in natural yoghurt demonstrated that greater than 98.3% of the culture was killed within 5 minutes at 30°C. Within 60 minutes no viable cells remained, (Figure 6).

In the case of cottage cheese inoculated with 10^4 cfu/ml *Listeria monocytogenes* 40% of the population was killed within 5 minutes at 30°C in the presence of a 10% lacticin 3147 powder. After 160 minutes only 14% of the population remained viable, (Figure 7).

The effect of 1, 5 and 10% concentrations of lacticin 3147 in powdered soup against *Bacillus cereus* at 30°C, demonstrated that following 24 hours incubation greater than a 99.9% kill was observed in the presence of the 5 and 10% lacticin 3147 powder concentrations. In the case of the 1% lacticin 3147 concentration 17% of the population survived, (Figure 8).

A similar study was carried out to determine the effect of 1, 5 and 10% concentrations of lacticin 3147 powder on the survival of *Listeria monocytogenes* Scott A in powdered soup. A 1% concentration of lacticin was ineffective at inhibiting Scott A within 24 hours, whereas at a 5% concentration greater than 10% of the population were inhibited. At a concentration of 10% greater than 40% of the culture was inhibited, (Figure 9).

From these results it can be seen that a powdered form of lacticin 3147 has indeed many applications in food safety for the control of food pathogens and spoilage organisms.

Effect of Hydrostatic Pressure

The use of hydrostatic pressure and lacticin 3147 treatments were evaluated in milk and whey with a view to combining both treatments for improving the quality of minimally processed dairy foods. The system was evaluated using two foodborne pathogens, *Staphylococcus aureus* ATCC6538 and *Listeria innocua* DPC1770. Trials against *Staph. aureus* ATCC6538 were performed using concentrated lacticin 3147 prepared from culture supernatant. Results demonstrated greater than an additive effect when both treatments were used in combination, for example, the combination of 250 MPa (2.2 log reduction) and lacticin 3147 (1 log reduction) resulted in more than 6 logs of kill (Fig. 10). Similar results were obtained when a foodgrade powdered form of lacticin 3147 (developed from a spray dried fermentation of reconstituted demineralised whey powder) was evaluated for the inactivation of *L. innocua* DPC1770 (Fig. 11). Furthermore, it was observed that treatment of lacticin 3147 preparations with pressures greater than 400 MPa yielded an increase in bacteriocin activity (equivalent to a doubling of activity). These results indicate that a combination of high pressure and lacticin 3147 may be suitable for improving the quality of minimally processed foods at lower hydrostatic pressure levels.

DISCUSSION

The development of a whey based bio-active food ingredient was achieved following investigations into lacticin 3147 production in different media. Lacticin 3147 activity was high in all of the dairy based media investigated, apart from whey protein concentrate (WPC35). A possible explanation for the low level of activity in the whey protein concentrate could be that bacteriocin activity fractionated into the pellet upon centrifugation, prior to assaying for activity. Two synthetic media were investigated for lacticin 3147 production, LM17 broth (20) and TY broth (15). Levels of lacticin 3147 activity in LM17 were comparable to dairy based media, but this is not unexpected, since this media was developed for the cultivation of lactococci. However, TY broth, in which low levels of lacticin 3147 activity was observed, was developed to yield optimal bacteriocin (enterocin 1146) production while minimising peptide levels in the medium (to eliminate peptides that may interfere with purification). For the development of a powder the use of the most cost effective growth media is obviously advantageous. Demineralized whey powder, a readily available and cost effective medium ($20 per 25 Kg) was investigated for the optimization of lacticin 3147 production. However, other suitable growth media could be used, as described above.

The effect of pH on bacteriocin production has been well documented, and for a number of bacteriocin-producing strains control of pH during growth results in higher bacteriocin titres (11, 14, 18). Lacticin 3147 activity increased dramatically when the pH of the growth media was held constant at pH 6.5, 5 highest bacteriocin titres and highest cell numbers were observed at this pH. Lowest bacteriocin titres and lowest cell numbers were observed when no pH control was imposed. Increased bacteriocin activity corresponded with increased cell numbers.

Once lacticin 3147 production had been optimized in 10% reconstituted demineralized whey powder a large-scale fermentation was set up to generate enough fermentate for spray drying. The production of an active spray dried powder demonstrated the resilience of the bacteriocin, to the extremes of the processing conditions. Activity was detected throughout the process and the final powder had an activity of 102,400 AU/g dry matter, equivalent to the activity present at the beginning of the process. This unexpected result is significant in that it suggests that no loss in activity occurred during production.

Assessment of the inhibitory activity of the bio-active powder demonstrated that it is capable of inhibiting both *L. monocytogenes* and *S. aureus* at pH 5 and at pH 7. In both cases the bio-active powder exhibited enhanced killing ability at neutral pH. This is a significant finding, since Nisaplin, a fermented food ingredient for extension of product shelf life and prevention of spoilage is known to be most effective at acidic pH (below pH 6.0). The development of a food ingredient capable of killing Gram-positive bacteria at neutral pH indicates that the lacticin 3147 powder may be suitable for incorporation into a wide range of foods, that hitherto had no opportunity for the prevention of food spoilage/pathogenesis apart from the inclusion of chemical preservatives.

The mechanism of action of lacticin 3147 has been elucidated (12). It induces cell death by permeabilising the membranes of sensitive cells through pore formation, allowing the efflux of K^+ ions and phosphate. This action results in the dissipation of the proton motive force, hydrolysis of intracellular ATP and ultimately leads to cell death. Energised cells are more susceptible to the action of lacticin 3147. Cells incubated in the presence of lacticin powder combined with 10mM glucose demonstrated slight increases in killing efficiency (apart from *S. aureus* 10 at pH 7, results not shown). This is in keeping with results reported by McAuliffe *et al.*, (12), where energised cells were observed to be more sensitive to lacticin 3147. Energised cells have a proton motive force which may favour the insertion of lacticin 3147 molecules into the membrane, as is the case with nisin, a lantibiotic pore former (7, 8).

The development of a powdered form of lacticin 3147 would allow it to be applied to a number of food systems. Since the existing lacticin 3147 powder has been developed from a demineralized whey powder, this powder has applications in all foods where demineralized whey powder is an existing ingredient. For example demineralized whey powder is incorporated into a number of foods including infant milk formulations. Results presented in this paper demonstrate the ability of this powder to effectively inactivate 99% of *L. monocytogenes* Scott A spiked into infant formula, where part of the infant milk powder had been substituted with the lacticin 3147 powder. Infant milk formulations are manufactured to the highest of standards and incidents of food-borne illness associated with such foods are rare. However more than many other foods infant milk formulas are susceptible to contamination through domestic contamination, putting the health of infants at risk. For this reason the inclusion of a lacticin 3147 enriched powder in such formulations may offer increased protection in the event of contamination, which would be beneficial to both producers and consumers.

For manufacturers already using demineralized whey powder as a food ingredient it should prove possible to substitute this powder (either partially or fully) with a bio-active demineralized whey powder to further safe guard food products from spoilage and pathogenic Gram-positive organisms. And indeed, for manufacturers who do not use demineralized whey powder as a food ingredient the inclusion of low levels of the bio-active powder could be sufficient to confer enhanced protection without affecting the sensory or functional characteristics of these foods. It is, however, also apparent that a spray-dried lacticin 3147 powder based on a medium other than whey powder, would be obtainable by this invention. Such a powder has potential for application as a substitute in areas where whey powder is not utilised, with the same beneficial effects.

SUMMARY

The broad-spectrum bacteriocin lacticin 3147, produced by *Lactococcus* lactis DPC3147, is inhibitory to a wide range of Gram-positive food spoilage and pathogenic organisms. A 10% solution of demineralized whey powder was fermented with DPC3147 at a constant pH of 6.5. The fermentate was spray dried and the resulting powder exhibited inhibitory activity. The ability of the lacticin 3147-enriched powder to inhibit *Listeria monocytogenes* Scott A and *Staphylococcus aureus* 10 was assessed in buffer at both acidic (pH 5) and neutral pH (pH 7). In addition, the ability of the powder to inhibit *L. monocytogenes* Scott A in an infant milk formulation was assessed. Resuspension of 8.3 log mid-exponential phase *L. monocytogenes* Scott A cells in a 10% solution of the lacticin 3147-enriched powder resulted in a 1000 fold reduction in viable cells at pH 5 and pH 7, after 3 hours at 30°C. In the case of *S. aureus* 10, resuspension of 2.5 x 10^7 mid-exponential phase cells in a 15% solution of the lacticin 3147-enriched powder at pH 5 resulted in only a 10 fold reduction in viable cell counts, compared to a 1000 fold reduction at pH 7, following incubation for 3 hours at 30°C. In an infant milk formulation the use of the lacticin 3147 powder resulted in greater than a 99% kill of *L. monocytogenes* within 3 hours at 30°C. Similarily, the lacticin 3147 powder was shown to be effective in inhibiting food spoilage in powdered soup, yoghurt and cottage cheese. Furthermore, the combination of hydrostatic pressure and lacticin 3147 causes increased killing making this an attractive method of preventing spoilage in minimally processed foodstuffs. Thus this bio-active lacticin 3147 food ingredient will find applications in many different foods, including those with pH close to neutrality.

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The words "comprises/comprising" and the words "having/including" when used herein with reference to the present invention are used to specify the presence of stated features, integers, steps or components but does not preclude the presence or addition of one or more other features, integers, steps, components or groups thereof.

Table 1. Lacticin 3147 activity in various media after overnight incubation at 30°C.

Growth Media	**Lacticin 3147 activity (AU/ml)**
Cheddar cheese whey	2560
Whole milk	2560
Reconstituted skimmed milk powder	1280
Reconstituted demineralised whey powder	1280
Whey protein concentrate (WPC35)	320
LM17	2560
TY broth	640

CLAIMS

1. A process for the production of spray-dried lacticin 3147 powder, for use as a food ingredient, comprising

(a) inoculating a medium with a lacticin 3147-producing strain of bacteria;

(b) fermenting the inoculated medium;

(c) adjusting the pH of the fermentation to pH 6.3 to 6.7;

(d) inactivating the bacterial fermentate;

(e) evaporating the fermentate of step (d).

2. A process as claimed in claim 1 wherein the medium is selected from milk or dairy-based powders including demineralized whey powder, reconstituted skimmed milk powder, whey protein concentrate powder, pasteurised whole milk, Cheddar cheese whey, yeast powders or synthetic laboratory-type media such as LM17 and TY broth.

3. A process as claimed in claim 1 or 2 wherein the concentrate of step (e) is cooled, seeded with lactose at about 0.1% w/w and allowed to crystallise at a cooling rate of about 1°C per hour.

4. A process as claimed in any preceding claim wherein the inoculated medium is fermented at about 30°C for about 6 to 24 hours.

5. A process as claimed in any preceding claim wherein the pH of the fermentation is adjusted to about pH 6.5

6. A process as claimed in any preceding claim whenever the fermentate is inactivated by pasteurisation or ultra-high temperature treatment.

7. A process as claimed in claim 6 wherein if the fermentate is pasteurised, it is pasteurised at about 72°C for about 15 minutes.

8. A process as claimed in any preceding claim wherein the fermentate of step (d) is evaporated to about 6°C to about 40% total solids.

9. A process as claimed in any preceding claim wherein the crystallised concentrate is spray-dried.

10. A process as claimed in any preceding claim substantially as described herein.

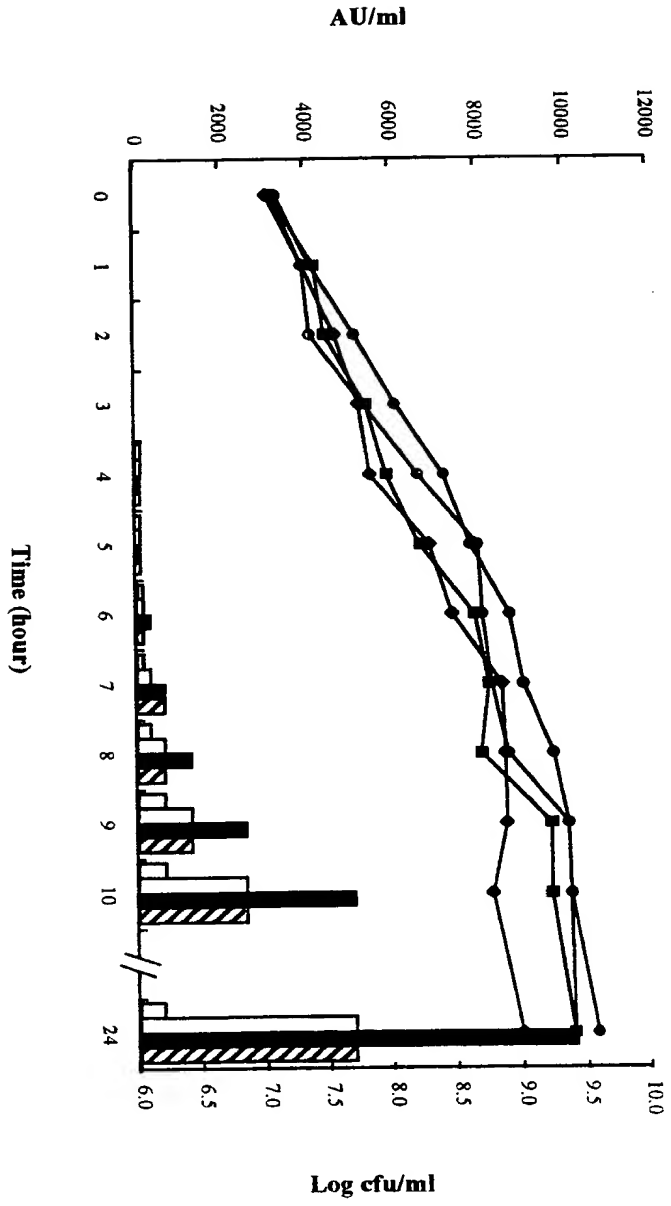
11. A spray-dried lacticin 3147 powder whenever produced by a process as claimed in any of claims 1 to 10.

12. Spray-dried lacticin 3147 having the ability to inhibit organisms which are not resistant to lacticin 3147, and preferably having an activity of greater than about 20,000 AU/ml, preferably about 30,000 AU/ml, more preferably about 40,240 AU/ml.

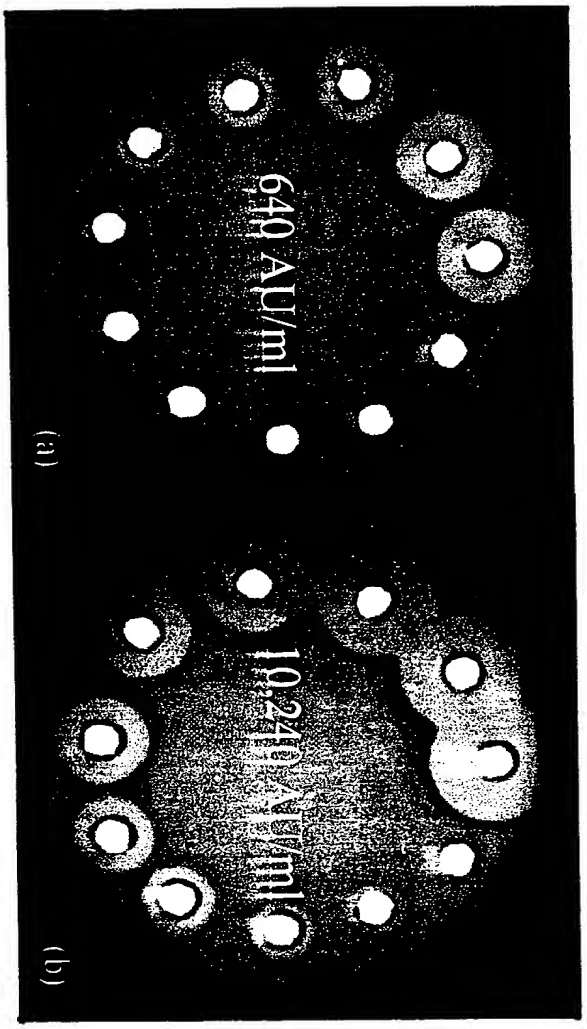
13. A food product comprising a spray-dried lacticin 3147 as claimed in claim 11 or claim 12 or as produced by a process as claimed in any of claims 1 to 10.

14. A food product as claimed in claim 12 which is selected from an infant milk formulation, a sauce, a mayonnaise, a dessert, a custard, a tinned food such as a vegetable or meat product, a yoghurt, a soup or a bakery product.

15. A food product as claimed in claim 13 and 14 which has been subjected to increased hydrostatic pressure, preferably in the range 150 to 800 MPa.


AU/ml
12000
10000
8000
6000
4000
2000
0
0 1 2 3 4 5 6 7 8 9 10 24
Time (hour)
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
Log cfu/ml

(A)


640 AU/ml
(a)
(b)

(B)

FIG. 1

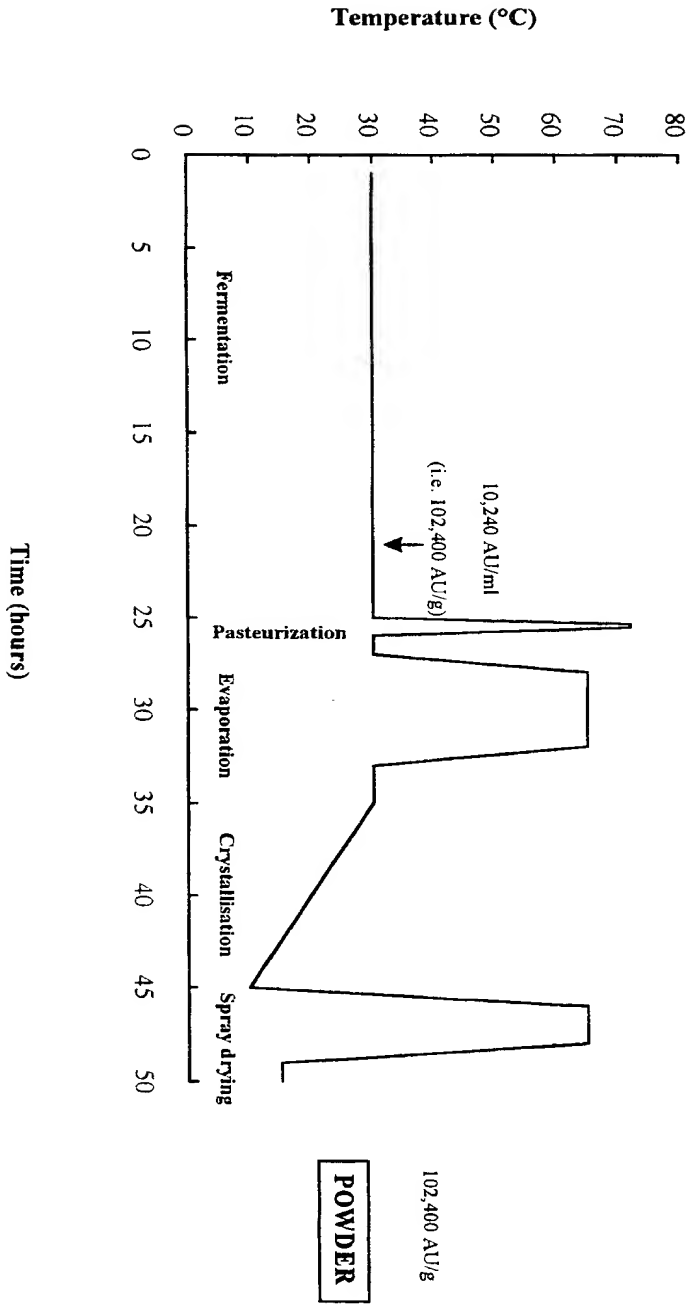

Temperature (°C)
0
10
20
30
40
50
60
70
80
0
5
10
15
20
25
30
35
40
45
50
Time (hours)
Fermentation
10,240 AU/ml
(i.e. 102,400 AU/g)
Pasteurization
Evaporation
Crystallisation
Spray drying
POWDER
102,400 AU/g

FIG. 2

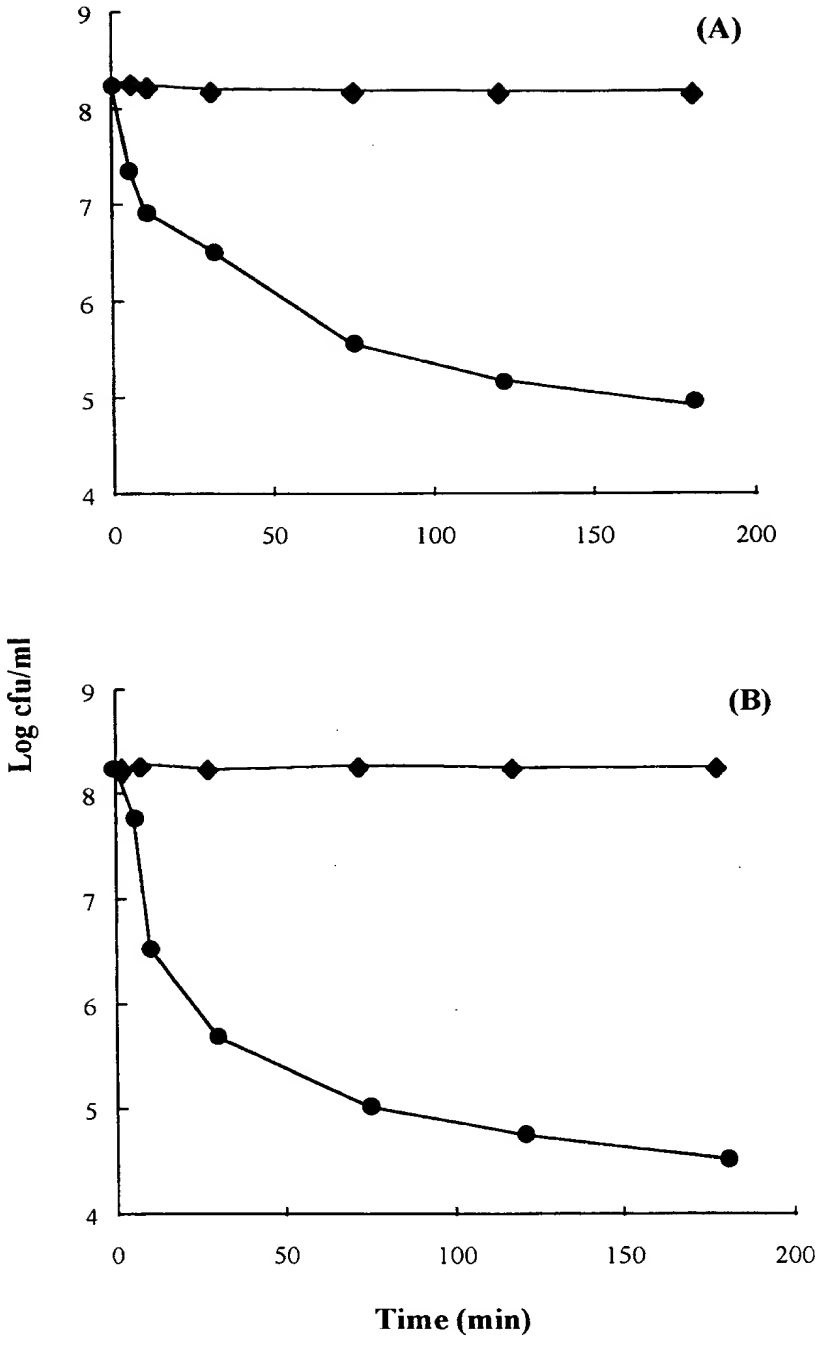


FIG. 3

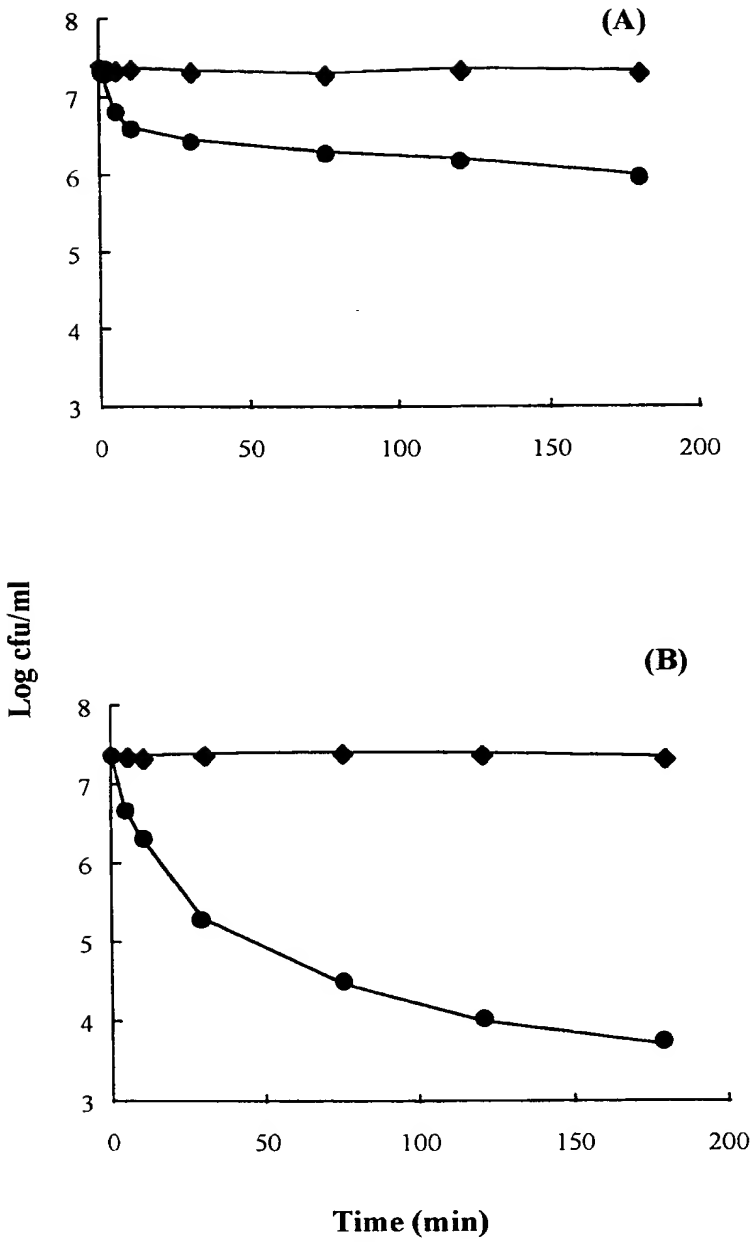

(A)
8
7
6
5
4
3
0
50
100
150
200
Log cfu/ml
(B)
8
7
6
5
4
3
0
50
100
150
200
Time (min)

FIG. 4

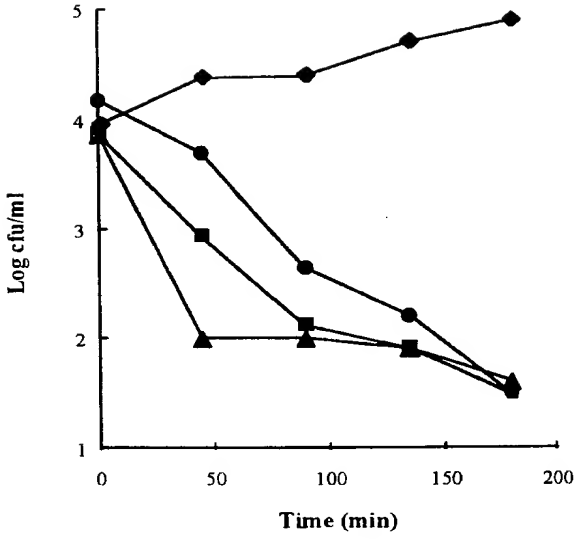
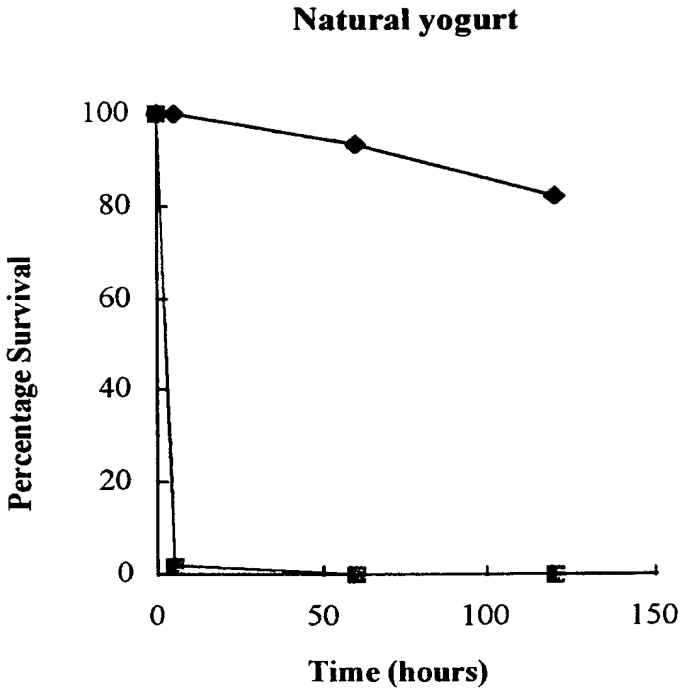


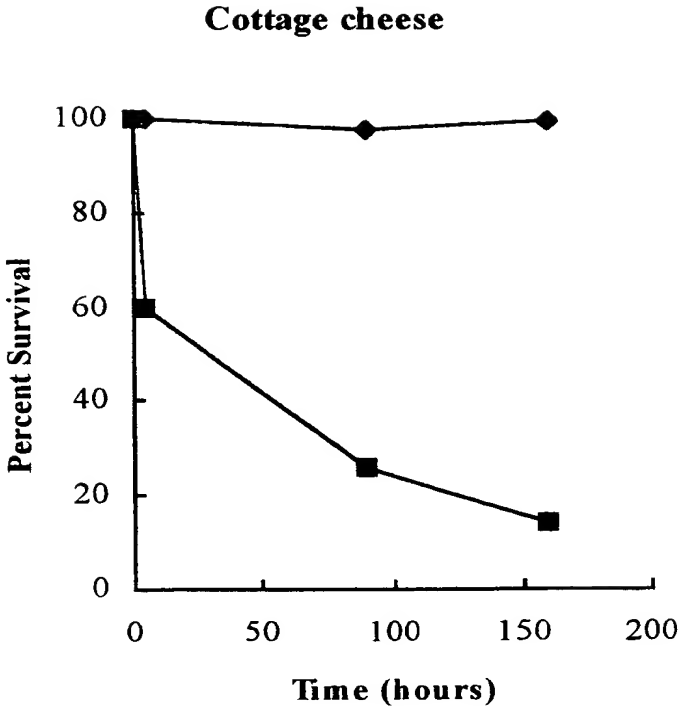
FIG. 5



Effect of lacticin 3147 powder (10%) on the viability of *Listeria monocytogenes* Scott A in yogurt. (◆) no lacticin 3147 added, (■) 10% lacticin 3147 added.

The 10% here refers to 10g lacticin 3147 powder added to 90g yogurt.

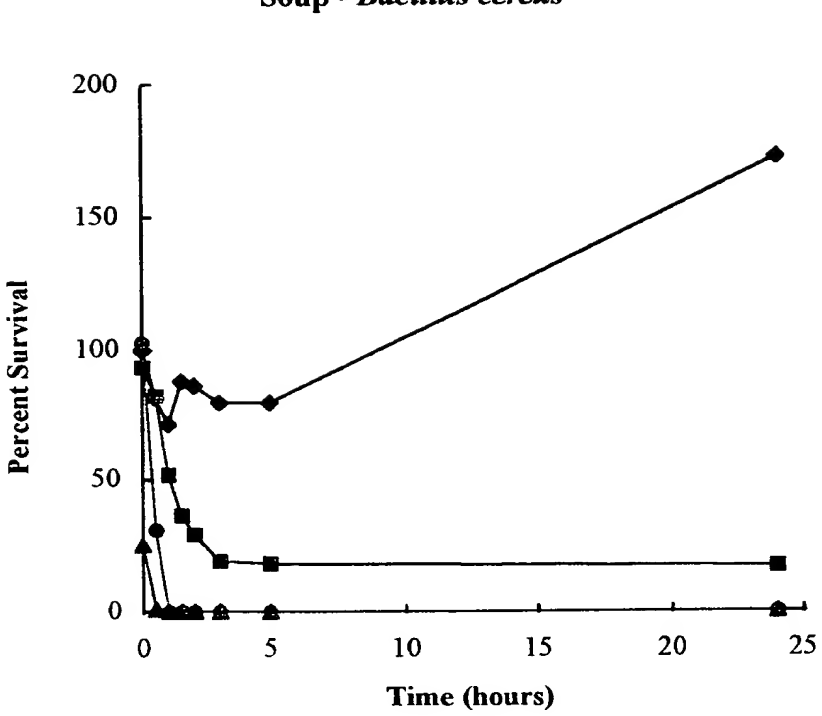
FIG. 6



Effect of lacticin 3147 powder (10%) on the viability of *Listeria monocytogenes* Scott A in cottage cheese. (◆) no lacticin 3147 added, (■) 10% lacticin 3147 added.

The 10% here refers to 10g lacticin 3147 powder added to 90g cottage cheese.

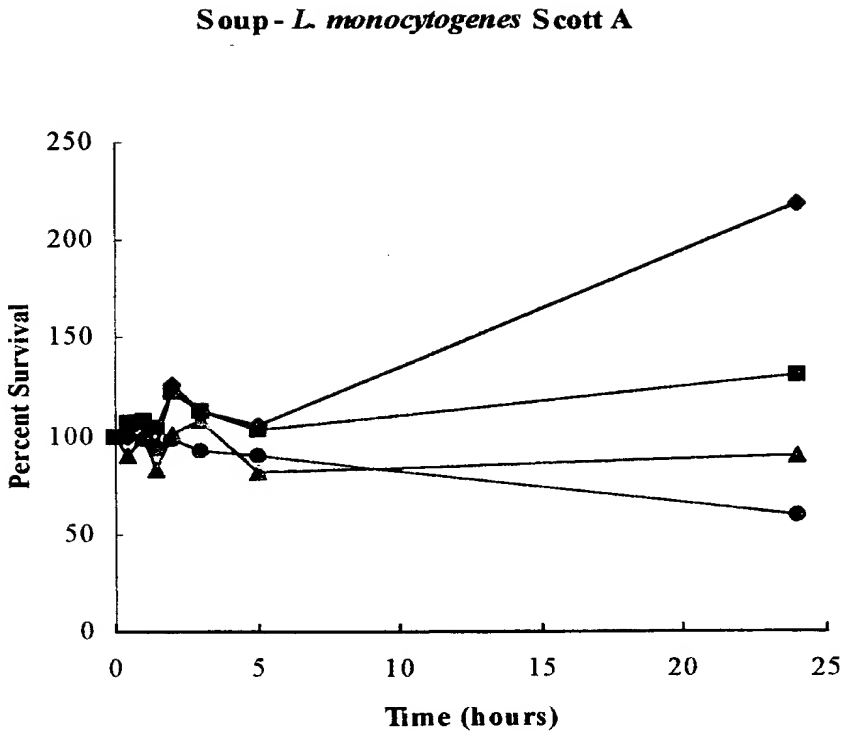
FIG. 7



Effect of lacticin 3147 powder (10%) on the viability of *Bacillus cereus* in (packet) soup.
(◆) no lacticin 3147 added,
(■) 1% lacticin 3147 added
(▲) 5% lacticin 3147 added
(●) 10% lacticin 3147 added

The 1, 5, 10% here refers to 1, 5 or 10g lacticin 3147 powder added to 99, 95 or 90g packet soup powder, then reconstituted to the manufacturers instructions.

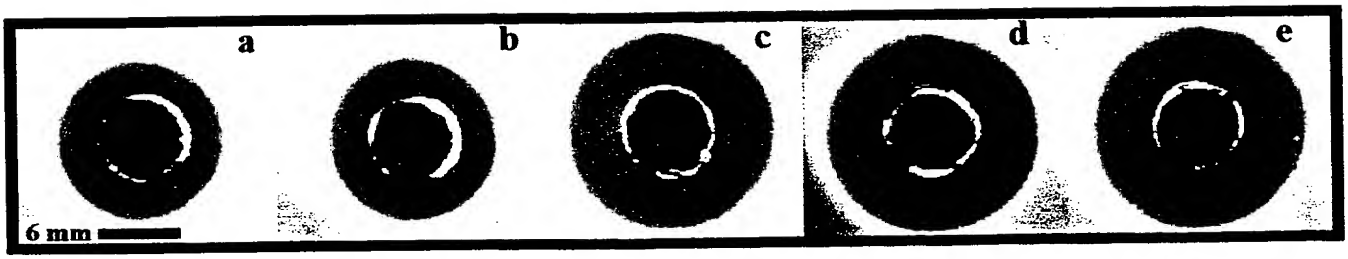
FIG. 8



Effect of lacticin 3147 powder (10%) on the viability of *Listeria monocytogenes* ScottA in (packet) soup.
(◆) no lacticin 3147 added,
(■) 1% lacticin 3147 added
(▲) 5% lacticin 3147 added
(●) 10% lacticin 3147 added

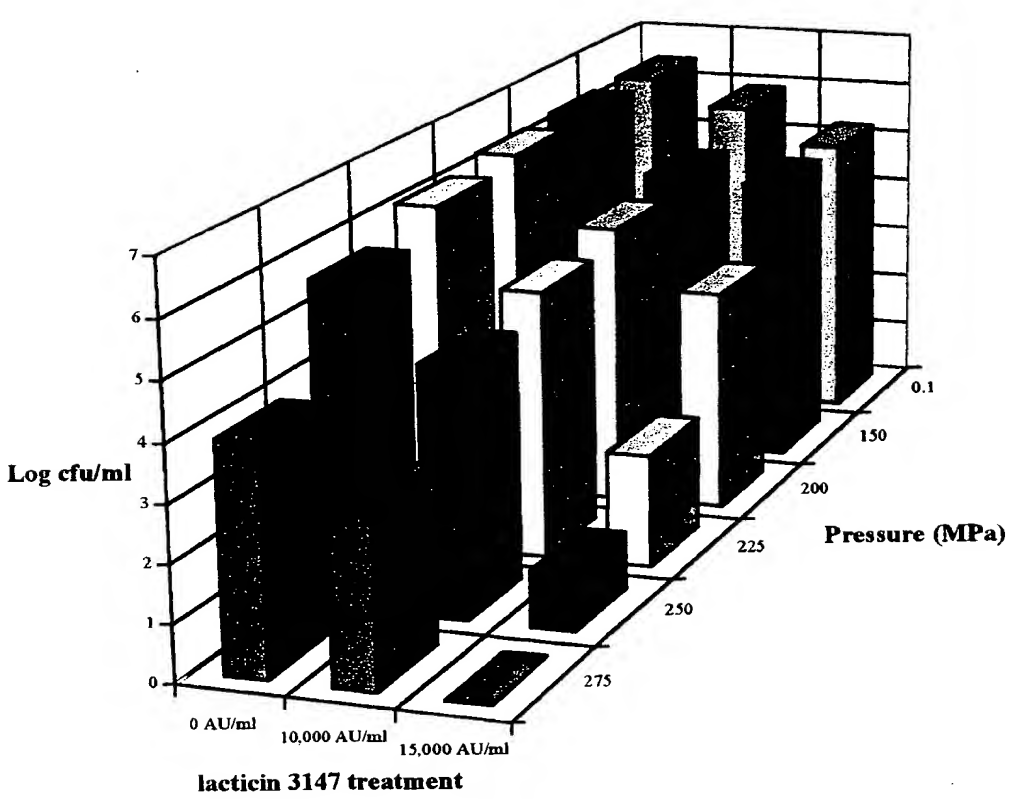
The 1, 5, 10% here refers to 1, 5 or 10g lacticin 3147 powder added to 99, 95 or 90g packet soup powder, then reconstituted to the manufacturers instructions.

FIG. 9



The effect of increasing pressures on the activity of lacticin 3147,
(a) atmospheric pressure, (b) 200 MPa, (c) 400 MPa, (d) 600 MPa and (e) 800 MPa.

FIG. 10



The effect of high pressure and lacticin 3147 on *L. innocua* DPC1770 viability.

FIG. 11

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IE 99/00058

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/315 A23L3/3463 A23C9/123 A23C9/158

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A23L A23C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	O. MCAULIFFE: "Lacticin 3147, a broad-spectrum bacteriocin which selectively dissipates the membrane potential" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 64, no. 2, 1998, pages 439-445, XP002119743 cited in the application page 439 -page 440 ---	1,2,5, 9-14
Y	DE 26 16 390 B (INSTYTUT PRZEMYSLU MLECZARSKIEGO) 6 October 1977 (1977-10-06) the whole document ---	1,2,5, 9-14
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 October 1999	09/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Desmedt, G

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE FSTA 'Online! INTERNATIONAL FOOD INFORMATION SERVICE (IFIS), FRANFURT/MAIN, DE PETERKOVA L ET AL: "Study of the manufacture of dried milk with high nisin content." Database accession no. 75-2-06-p1345 XP002119744 abstract & PRUMYSL POTRAVIN, vol. 25, no. 12, 1974, pages 377-379, Vyzkumny Ustav Mlekarensky, Prague, Czechoslovakia ---	1,2,5, 10-15
A	RYAN M P ET AL: "AN APPLICATION IN CHEDDAR CHEESE MANUFACTURE FOR A STRAIN OF LACTOCOCCUS LACTIS PRODUCING A NOVEL BROAD-SPECTRUM BACTERIOCIN, LACTICIN 3147" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 62, no. 2, 1 February 1996 (1996-02-01), pages 612-619, XP000578691 ISSN: 0099-2240 cited in the application page 612 -page 613 ---	1
A	WO 96 32482 A (RYAN MAIRE PHILIPPA ;REA MARY CLARE (IE); ROSS REYNOLDS PAUL (IE);) 17 October 1996 (1996-10-17) cited in the application -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IE 99/00058

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
DE 2616390	B	06-10-1977	NONE		
WO 9632482	A	17-10-1996	IE	950269 A	16-10-1996
			AU	5408196 A	30-10-1996
			EP	0821736 A	04-02-1998
			NZ	305941 A	28-01-1999

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PATENT COOPERATION TREATY

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WIPO PCT

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
PM111PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IE99/00058	22/06/1999	22/06/1998
International Patent Classification (IPC) or national classification and IPC C07K14/315		
Applicant TEAGASC, THE AGRICULTURE AND FOOD... et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Auth rity and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
20/01/2000	09.11.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Halle, F Telephone No. +49 89 2399 8537

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IE99/00058

I. Basis of the r port

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-19 as originally filed

Claims, No.:

1-15 as originally filed

Drawings, sheets:

1/11-11/11 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 10.

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 10 are so unclear that no meaningful opinion could be formed (*specify*):

see separate sheet

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-9
	No:	Claims	11-15
Inventive step (IS)	Yes:	Claims	1-9
	No:	Claims	11-15
Industrial applicability (IA)	Yes:	Claims	1-9,11-15
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Point III

The subject-matter of claim 10, as presently formulated, is too vague for an objective examination to be carried out thereon.

Point V

1. Reference is made to the following documents:

D1: Appl. Envir. Microbiology 62(2), 1996, p. 612-619 (cited in the application)
D2: WO-A-96 32482 (cited in the application)
D3: DE 26 16 390
D4: Database FSTA, Accession No. 75-2-06-p1345

2.1 The invention relates, in particular, to a spray-dried bacteriocin powder with antibacterial activity. The powder contains " lacticin 3147" which inhibits Listeria monocytogenes in Cottage cheese.

In the following report, it is at first referred to the product claims.

Product claims 11-15

2.2 Having regard to the prior art documents D1 or D2, the subject-matter of claims 11 and 12 does not appear to be novel. Lacticin 3147 is a well known bacteriocin which has been described in cheese making processes, see for example D1, page 616, left column, last paragraph. Furthermore, it is to be noted that a known product cannot be rendered novel by a new process. Even if lacticin 3147 is spray-dried it is still the same compound. Therefore, as presently claimed, the preparation of a known product in a specific physical form does not render novel said known product.

2.3 The subject-matter of claims 13-15 is also anticipated by the disclosure of D1 which discloses Cheddar cheese preparations containing lacticin 3147. In this case lacticin is produced by the strain Lactococcus lactis which has been added to the cheese preparation. The final cheese product is a food product containing

lacticin. Therefore, the subject-matter of claims 13-15 is anticipated by the disclosure of D1.

2.4 Furthermore, claims 11-15 would not appear to contain any inventive subject-matter over the prior art documents D2, D3 and D4. Indeed, an other bacteriocin, termed nisin, is used in dried food (cf. D3 and D4). Furthermore, nisin and lacticin 3147 have similar microbial inhibiting properties (cf. D2). On the basis of these similar properties, the skilled person would obviously be inclined to also use lacticin 3147 in dried food preparations.

Process claims 1-10

2.5 Having regard to the cited prior art, the subject-matter of claims 1-9 (claim 10 is unclear, see item 3.1 below) would appear to be novel (Article 33(2) PCT); the prior art documents do not disclose the production of a spray-dried lacticin 3147 powder.

2.6 Furthermore, the subject-matter of claims 1-9 would appear to involve an inventive step (Article 33(3) PCT). Although the prior art documents D3 and D4 refer to a similar bacteriocin (nisin, see item 2.4 above) contained in dried milk, a specific process for the production of a spray-dried nisin powder is not described in said prior art documents. Moreover, in the presently claimed process, the bacteriocin activity of lacticin 3147 is not destroyed by the spray-drying process. Therefore, this process may be considered as an advantageous alternative process for the production of lacticin in the form of a biologically-active powder which may be applied as a food ingredient in a variety of foods.

Point VIII

3.1 The subject-matter of claim 10 is unclear and should be amended (Article 6 PCT).

3.2 The prior art documents D1 and D2 are not mentioned in the description (Rule 5.1(a)(ii) PCT).

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PATENT COOPERATION TREATY

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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
PM111PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/IE 99/ 00058	22/06/1999	22/06/1998

Applicant

TEAGASC, THE AGRICULTURE AND FOOD DEVELOP. et al.

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established, according to Rul 38.2(b), by this Authority as it appears in Box III. The applicant may, within ne month from th date of mailing of this international search report, submit comments to this Authority.

6. The figure of th **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by th applicant.

   [ ] because th applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of th figures.

Box III TEXT OF THE ABSTRACT (Continuation of item 5 of the first sheet)

Line 5 : after "hydrostatic pressure" add
"The process comprises: inoculating a medium with a lacticin 3147-producing strain of bacteria, fermenting the inoculated medium, adjusting the Ph of the fermentation to 6.3-6.7, inactivating the bacterial fermentate and evaporating the fermentate".

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IE 99/00058

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/315 A23L3/3463 A23C9/123 A23C9/158

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A23L A23C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	O. MCAULIFFE: "Lacticin 3147, a broad-spectrum bacteriocin which selectively dissipates the membrane potential" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 64, no. 2, 1998, pages 439-445, XP002119743 cited in the application page 439 -page 440 ---	1,2,5, 9-14
Y	DE 26 16 390 B (INSTYTUT PRZEMYSLU MLECZARSKIEGO) 6 October 1977 (1977-10-06) the whole document --- -/--	1,2,5, 9-14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 October 1999	09/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Desmedt, G

1

Form PCT/ISA/210 (second sheet) (July 1992)

International Application No
PCT/IE 99/00058

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE FSTA 'Online! INTERNATIONAL FOOD INFORMATION SERVICE (IFIS), FRANFURT/MAIN, DE PETERKOVA L ET AL: "Study of the manufacture of dried milk with high nisin content." Database accession no. 75-2-06-p1345 XP002119744 abstract & PRUMYSL POTRAVIN, vol. 25, no. 12, 1974, pages 377-379, Vyzkumny Ustav Mlekarensky, Prague, Czechoslovakia	1,2,5, 10-15
A	RYAN M P ET AL: "AN APPLICATION IN CHEDDAR CHEESE MANUFACTURE FOR A STRAIN OF LACTOCOCCUS LACTIS PRODUCING A NOVEL BROAD-SPECTRUM BACTERIOCIN, LACTICIN 3147" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 62, no. 2, 1 February 1996 (1996-02-01), pages 612-619, XP000578691 ISSN: 0099-2240 cited in the application page 612 -page 613	1
A	WO 96 32482 A (RYAN MAIRE PHILIPPA ;REA MARY CLARE (IE); ROSS REYNOLDS PAUL (IE);) 17 October 1996 (1996-10-17) cited in the application	

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INTERNATIONAL SEARCH REPORT

[redacted]ation on patent family members

[redacted]ational Application No
[redacted]CT/IE 99/00058

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
DE 2616390	B	06-10-1977	NONE		
WO 9632482	A	17-10-1996	IE	950269 A	16-10-1996
			AU	5408196 A	30-10-1996
			EP	0821736 A	04-02-1998
			NZ	305941 A	28-01-1999

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 18 February 2000 (18.02.00)	
International application No. PCT/IE99/00058	**Applicant's or agent's file reference** PM111PCT
International filing date (day/month/year) 22 June 1999 (22.06.99)	**Priority date** (day/month/year) 22 June 1998 (22.06.98)
Applicant ROSS, Reynolds, Paul et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

20 January 2000 (20.01.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Jean-Marc Vivet

Telephone No.: (41-22) 338.83.38